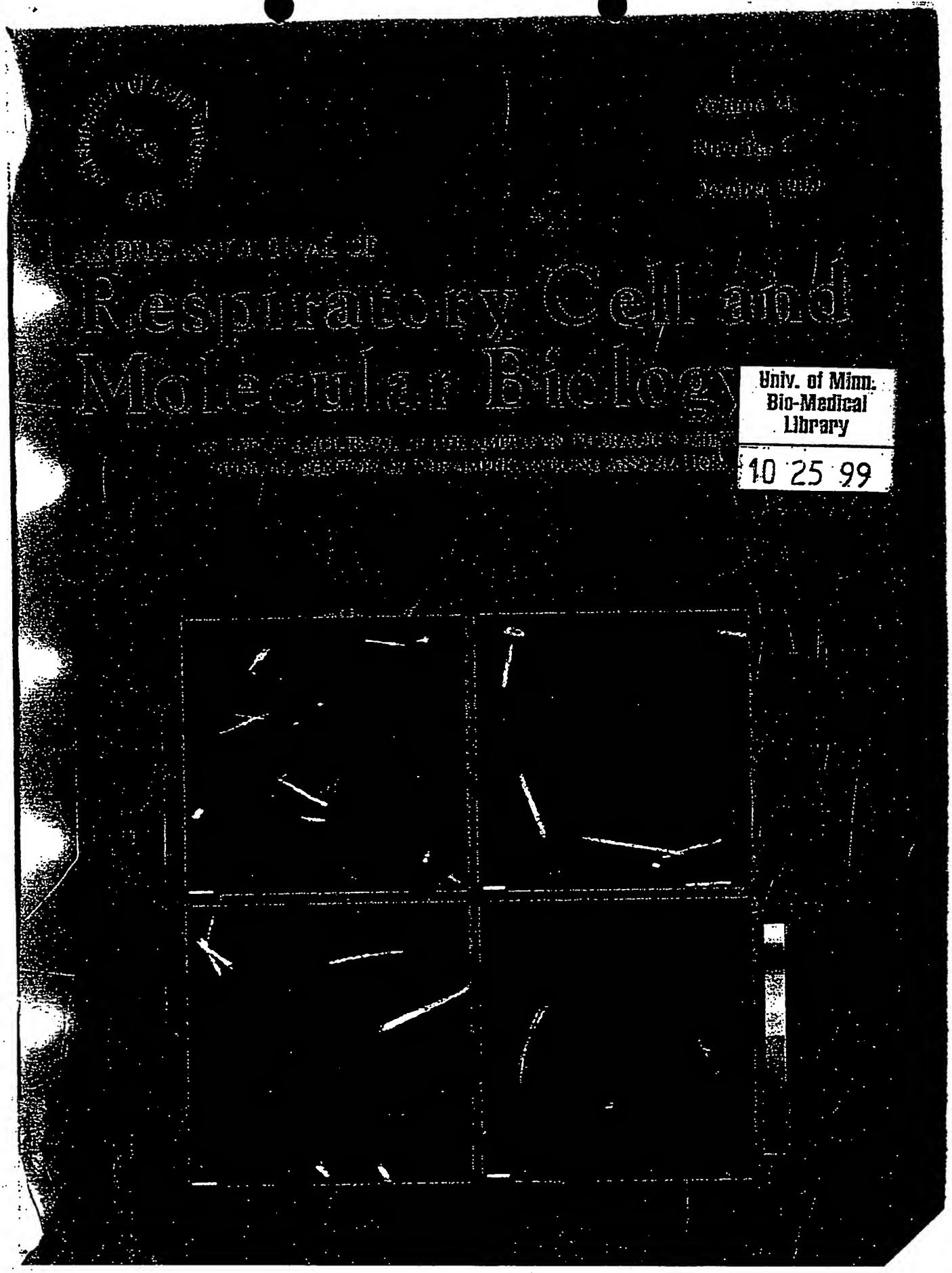
EXHIBIT 5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CD28 Interactions with Either CD80 or CD86 Are Sufficient to Induce Allergic Airway Inflammation in Mice

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Previous studies have shown that the pan CD28/cytotoxic T lymphocyte antigen (CTL)A-4 antagonist CTLA4 immunoglobulin (Ig) inhibits eosinophilic airway inflammation in *Schistosoma mansoni*–sensitized and airway-challenged mice. In the present study, the importance of CD28 as well as the individual roles of CD80 and CD86 were examined in this system using wild-type and CD28 knockout (KO) mice. Unlike wild-type controls, CD28KO mice did not produce systemic IgE or eosinophilic airway inflammation after antigen challenge. However, a lymphocytic infiltrate and continued production of interferon-γ was observed in these animals. Thus, CD28 is not essential for the initial recruitment of lymphocytes into antigen-challenged airways but critically regulates the allergic T-helper 2 phenotype. We next determined by polymerase chain reaction and flow cytometry that CD80 and CD86 molecules are constitutively expressed in the naive murine lung and on eosinophils in the allergic lung, suggesting a potential important role for both ligands in the development of asthma. Combined anti-CD80/anti-CD86 treatment throughout the antigen challenge period fully blocked the development of allergic airways, whereas a partial reduction was observed in mice treated with either anti-CD80 or anti-CD86 antibody alone. However, only anti-CD86 blocked systemic IgE production. Therefore, signaling through either CD80 or CD86 is sufficient to generate a partial local allergic response, whereas CD86 costimulation is essential to induce systemic allergic (IgE) reactions. Finally, combined anti-B7 monoclonal antibody treatment after sensitization reduced airway eosinophilia and interleukin (IL)-4/IL-5 cytokine secretion consistent with an ongoing role for CD28/B7 interactions in the effector phase of the disease. These results emphasize the importance of differential B7 expression on different cells and in different organs on subsequent CD28/B7-mediated immune events, including the potential for CD28/B7 blockade in the treatment of atopic airway disease in people. Mathur, M., K. Herrmann, Y. Qin, F. Gulmen, X. Li, R. Krimins, J. Weinstock, D. Elliott, J. A. Bluestone, and P. Padrid. 1999. CD28 interactions with either CD80 or CD86 are sufficient to induce allergic airway inflammation in mice. Am. J. Respir. Cell Mol. Biol. 21:498–509.

Atopic asthma is a disorder characterized clinically by spontaneous airflow limitation and nonspecific airway hyperresponsiveness. The mechanism(s) that underlie these pathologic clinical findings includes airway inflammation with eosinophilic infiltration of airway epithelium and submucosa (1). The generation of eosinophilic airway inflammation appears to be dependent on cytokine signals delivered by activated T lymphocytes (2–4). It has been established that full activation of T lymphocytes to direct the activation and migration of airway eosinophils requires two signals. The first signal occurs following the interaction of polymorphic T-cell receptors with the major histocompatibility complex peptide complex on potent antigen-presenting cells (APCs), including B cells, macrophages, and dendritic cells. This engagement initiates a cascade of biochemical signals to initiate the transcription of important effector proteins such as interleukin (IL)-2 and inflammatory cytokines such as interferon (IFN)-γ, and the development of cytolytic effector molecules. Complete T-cell activation and differen-

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Abbreviations: antigen-presenting cell, APC; bronchoalveolar lavage fluid, BALF; CD28-deficient, CD28KO; cytotoxic T lymphocyte antigen, CTL; diaminobenzidine tetrahydrochloride, DAB; experimental autoimmune encephalitis, EAE; enzyme-linked immunosorbent assay, ELISA; fluorescence-activated cell sorter, FACS; fluoresceln isothiocyanate, FITC; hematoxylin and eosin, H&E; interferon, IFN; immunoglobulin, Ig; interleukin, IL; knockout, KO; monoclonal antibody, mAb; median fluorescence intensity, MFI; messenger RNA, mRNA; polymerase chain reaction, PCR; sensitized and challenged, SCH; soluble egg antigen, SEA; T-helper, Th.

tiation, however, requires a second, noncognate set of interactions, the so-called costimulatory stimulus. The best-studied costimulatory stimulus involves the CD28/B7 family of molecules. CD28 is a cell-surface glycoprotein homodimer expressed on the majority of functional T cells. Both CD28 and the homologous family member cytotoxic T lymphocyte antigen (CTLA)-4 bind to the same B7 family of coreceptors, of which there are at least two members, CD80 and CD86 (5). These B7 molecules are differentially expressed on distinct tissues, have different requirements for induction, and exist in multiple forms with varying affinities for the CD28 and CTLA-4 receptors. Previous studies have shown that CD28 ligation regulates cell-cycle progression, cell survival, and cytokine and chemokine production (5).

Recently, treatment of mice with a soluble CD28/CTLA-4 antagonist, CTLA4 immunoglobulin (Ig) (a chimeric fusion protein consisting of the extracellular domain of CTLA-4 and the hinge, CH2, and CH3 regions of IgG), has been shown to inhibit the development of pathologic changes in airway structure and function in a number of murine models of asthma (6–9). We have shown that administration of CTLA4Ig after primary immunization with *Schistosoma mansoni* antigen can cause immune deviation from a primarily T-helper (Th)2–like response toward a primarily Th1-like response (8). Because CTLA4Ig inhibits the interaction of both CD28 and CTLA-4 to both CD80 and CD86, the functionally relevant interaction (i.e., CD28 or CTLA-4) and the specific roles of individual B7 molecules in the induction of T lymphocyte–mediated asthmatic airway inflammation remain unclear. In particular, the importance of CD28 in generating lymphocyte effector functions has recently been challenged by studies in CD28 "knockout" (KO) mice that are nevertheless capable of rejecting transplanted cardiac allografts in a T cell–specific manner (10, 11). Additionally, a number of cell types within the allergic lung can express B7 molecules, including T and B lymphocytes, macrophages, dendritic cells, epithelial cells, and eosinophils. However, it is not clear whether one or a combination of these cells plays a dominant role in B7 presentation in the allergic lung. An independent role for CD28/CD80 or CD28/CD86 ligation in generating the allergic airway phenotype has not been conclusively demonstrated. In the present study, we compared the effect of *S. mansoni* sensitization and challenge in wild-type mice, CD28KO mice, and mice treated with monoclonal antibodies (mAbs) to CD80 and CD86 during the entire antigen sensitization and challenge period or only during the period of antigen challenge. These studies suggest that CD28, not CTLA-4, is the essential B7 receptor for the induction and progression of Th2-mediated allergic airway inflammation in this murine model. Moreover, unlike peripheral T-cell responses, including those that control IgE production and graft rejection, CD80 and CD86 ligation provides equivalent critical costimulatory signals in response to the allergen. These results are consistent with the constitutive expression and kinetics of expression of CD80 and CD86 locally within lung in this animal model. This may be due to continued antigen stimulation in this tissue, including preferential expression of CD80 on eosinophils, a cell type that does not play a role in presentation of costimulatory molecules in nonallergic tissues. Finally, the ability of the combination of anti-CD80 and anti-CD86 mAb treatment to diminish allergic airway disease in mice after antigen sensitization suggests a potential therapeutic role for this treatment in humans with atopic asthma.

Materials and Methods

Animals

Female C57BL/6 (B6) mice (6 to 10 wk old) were purchased from Harlan Sprague-Dawley and housed in a specific pathogen–free facility maintained by the University of Chicago Animal Resources Center. CD28-deficient (CD28KO) mice were generated as previously described (12) and were bred onto a B6 background. The studies reported here conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Antibodies

Hamster-antimouse CD80 (13) and rat-antimouse CD86 (14) antibodies were produced at the University of Chicago in a high-density bioreactor (Endotronics Corp., Coons River, MN). Antibodies were purified as previously described (14, 15). Both antibodies were analyzed for binding specificity based on staining of CD80 and CD86 transfectants, respectively. As described later, mice were injected with either or both mAbs (50 μg/mouse, intraperitoneally) every other day beginning on Day 0 and throughout the treatment period. Control animals were treated with the relevant isotype control Ig (hamster or rat IgG; Southern Biotech, Birmingham, AL). Mouse CTLA4Ig was a generous gift from Genetics Institute (Cambridge, MA). CTLA4Ig was administered to a subset of CD28KO mice (50 μg/mouse, intraperitoneally) every other day beginning on Day 0 and throughout the treatment period. Anti-CD3 (145-2C11) was produced as previously described (16) and purified by passage over a protein A–coupled sepharose column. Anti-CD25, CD44, CD45, CD69, B220, and Thy1.2 used in fluorescence-activated cell sorter (FACS) analyses were obtained from PharMingen (San Diego, CA).

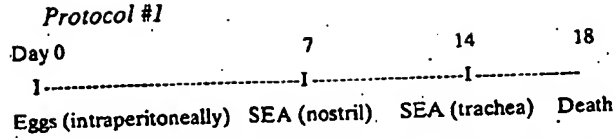
S. mansoni Eggs and Antigen

S. mansoni eggs were isolated and purified and soluble egg antigen (SEA) was produced as previously described (17). Eggs were stored at −70°C in 1.7% saline before use.

Antigen Sensitization and Challenge

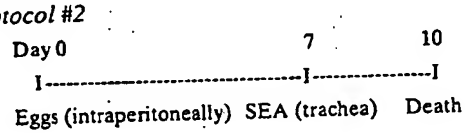
Two protocols were used in the following studies. In the first protocol, mice were immunized intraperitoneally with 5,000 isolated *S. mansoni* eggs at on Day 0. On Days 7 and 14 mice received 10 μg of SEA intranasally and intratracheally, respectively. Control animals were sensitized and challenged in the same manner with saline instead of eggs and SEA. Animals were killed 4 d after the intratracheal injection of SEA.

Protocol #1



As previously described, this protocol consistently and reliably induces maximal airway tissue eosinophilia, airway hyperresponsiveness to methacholine, and secretion of reproducible amounts of IL-5 into bronchoalveolar lavage fluid (BALF) (8). However, utilization of two airway challenges in Protocol #1 made it more difficult to evaluate the effect(s) of anti-B7 treatment in systemically sensitized mice before local antigen challenge. Therefore, Protocol #1 was modified so that airway inflammation was induced following only a single airway antigen challenge. In this second protocol, 5,000 eggs were injected intraperitoneally at Day 0 and 10 μg of SEA was injected intratracheally at Day 7. Control animals were sensitized and challenged in the same way with saline instead of eggs and SEA. Animals were killed 3 d after the intratracheal injection of SEA.

Protocol #2



Instrumentation

Mice were anesthetized by intraperitoneal injection of ketamine HCl and xylazine HCl. The trachea was cannulated with a 20-gauge, 1-cm metal needle, and the jugular vein was cannulated with P-10 tubing.

BAL

BAL was performed by injecting 0.8 ml of ice-cold phosphate-buffered saline (PBS) through the tracheal cannula and following it with gentle aspiration. This procedure was repeated three additional times. Fluids from all four lavages were pooled for maximum cell recovery. Cells were stained with trypan blue to determine viability, and total nucleated cells counts were determined using a Neubauer hemocytometer. Cytocentrifuge preparations were made using a cytocentrifuge (Shandon Southern Instruments, Sewickley, PA) set for 700 × *g* for 5 min. Cytospin slides were fixed and stained using Diff-Quik (American Scientific Products, McGaw Park, IL). Differential cell counts were determined by counting a minimum of 300 cells/slide, using standard morphologic criteria. Whole blood was withdrawn from the jugular catheter for determination of IgE.

Isolation and Stimulation of Lung Lymphocytes

Lungs from individual mice were digested for approximately 1 h in a buffer solution containing 850 U/ml hyaluronidase, 500 U/ml DNase I, and 1 mg/ml collagenase. Undigested tissue was allowed to settle and the resulting slurry was passed through a 55-μm Nytex filter. Erythrocytes were lysed and the remaining cells were washed three times in RPMI 1640 with 10% fetal calf serum (FCS). These washed cells were subsequently overlaid onto a Percoll gradient (50 to 70%). Cells within the 50 to 70% interface were aspirated and washed in complete media. An aliquot of these cells was subsequently stained with an anti-CD3 (145-2C11) mAb and analyzed by FACS to determine relative and absolute numbers of lymphocytes.

Proliferation Assay

T cells were separated from whole lung homogenates harvested from sensitized and challenged (SCH) CD28KO mice (including a group treated with CTLA4Ig) using magnetic cell sorting techniques (MACS; Miltenyi Corp., Gladbach, Germany). Cells were cultured in 96-well flat-bottomed plates coated with anti-CD3 (145-2C11, 1.0 μg/ml) or SEA (10 μg/ml) using 2×10^5 cells/well plus an equal number of irradiated spleen cells (final concentration: 2×10^6 cells/ml) in Dulbecco's modified Eagle's medium containing 10% FCS, 2 mM glutamine, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and gentamicin sulfate (5 μg/ml) at 37°C in 5% CO_2. Cells were pulsed 18 h before harvest with 1 μCi (methyl-[^{3}H]thymidine) (37 kBq; DuPont, Boston, MA) per well. The cells were harvested onto filters and the radioactivity on the dried filters was measured in a liquid scintillation counter. Incorporation during the last 18 h of culture (counts per minute) was used as an index of proliferation.

Reverse Transcriptase/Polymerase Chain Reaction Detection of CD80 and CD86 Messenger RNA Expression in Murine Lung Tissue

Many cells within murine lungs can potentially process and/or present antigen, including "professional" APCs (B cells, alveolar macrophages, dendritic cells) and nontraditional APCs (epithelial cells, eosinophils). Importantly, it has not yet been determined which one or what combination of these APCs plays a dominant role in T-lymphocyte activation in human asthma or in mouse models of asthma. Therefore, we evaluated the relative expression of messenger RNA (mRNA) for both CD80 and CD86 from all collagenase-digested lung cells at baseline, after sensitization but before challenge and at 12, 24, 48, and 72 h after SEA challenge in sensitized mice. Total RNA was isolated from 100 mg lung tissue from naive and sensitized mice using standard methodology (RNA "STAT-60" reagent; Tel-Test Inc., Friendswood, TX). RNA was treated with DNase I as follows: 50 μg RNA, 3 μl 10× DNase I digestion buffer, and 3 μl DNase I (1 U/μl; GIBCO BRL, Grand Island, NY), with diethylpyrocarbonate H_2O to a total volume of 30 μl, incubated at room temperature for 10 min. The reaction was stopped by adding 3 μl 0.5 mM ethylenediaminetetraacetic acid, followed by a 10-min incubation at 65°C. Samples were purified by phenol:chloroform:isoamyl alcohol extraction.

A total of 1 μg of RNA from each of the time points was transcribed by using 1 μg of MuLV Reverse Transcriptase in a total volume of 20 μl at 42°C for 15 min, followed by polymerase chain reaction (PCR) amplification. PCR reaction conditions were as follows: 94°C/15 s, 57°C/15 s, and 72°C/30 s, followed by a final extension step at 72°C/7 min.

The primers used in PCR were β-actin: 5′ primer 5′-ACCAGGGTGTGATGGTGGGAATGGG-3′, 3′ primer 5′-TTGCTGATCCACATCTGCTGGAAGG-3′; CD80: 5′ primer 5′-TGCTGTCTGTCATTGCTGGGAAACT-3′,

3′ primer 5′-CCCAGGTGAAGTCCTCTGACACGTG-3′; and CD86: 5′ primer 5′-TCCAGAACTTACGGAAG-CACCCACG-3′, 3′ primer 5′-CAGGTTCACTGAAGT-TGGCGATCAC-3′.

A total of 15 μl of each PCR product was electrophoresed on a 2% agarose gel without loading buffer. The gel was then stained in ethidium bromide media for 20 min, soaked in distilled H_2O for 10 min, and photographed with a Kodak DC120 digital camera under ultraviolet light. The digital images were analyzed with Kodak Digital Science 1D image analysis software (Kodak, Rochester, NY) and the net intensity of each band was determined. The relative intensity of β-actin complementary DNA was detected as a positive control for each mRNA sample.

Surface Expression of B7 Molecules on Lung Cells

Cell-surface expression of CD80 and CD86 on collagenase-digested murine lung cells at equivalent time points was determined as described for mRNA studies. Cells were washed with standard FACS buffer (PBS, 0.1% sodium azide, and 0.5% bovine serum albumin, pH 7) and incubated with mAbs specific for CD80 or CD86 (1:160 dilution for both mAbs) at a final concentration of 10^5 cells/well. Propidium iodide staining was performed to exclude dead cells from analysis. A total of 5 to 10 × 10^3 cells was analyzed for each monoclonal antibody using Lysis II software. Discrete cell populations identified in scatter plots were sorted, cytocentrifuged, and deposited on glass slides (Shandon Southern Instruments), stained with Diff-Quik or Wright stain, and identified based on typical morphologic criteria and staining characteristics. Eosinophils were specifically identified on the basis of the presence of discrete eosin-positive stained granules.

Enzyme-Linked Immunosorbent Assay for Determination of IL-4, IL-5, IFN-γ, and IgE

Cytokines in BALF (IL-4, IL-5, and IFN-γ) and IgE in serum were detected by commercially available enzyme-linked immunosorbent assay (ELISA) kits (IFN-γ and IL-4: Endogen, Cambridge, MA; IL-5 and IgE: PharMingen). The lower limits of detection for IL-4, IL-5, IFN-γ, and IgE were 10, 10, and 160 pg/ml, and 167 ng/ml, respectively.

Histology

Lungs from mice randomly chosen from all groups were removed from the chest cavity and fixed by injection of 10% buffered formalin (1.0 ml) into the tracheal cannula at a pressure of 20 cm H_2O, and immersed in formalin for 24 h. All lobes were sectioned sagittally, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E) for routine analysis.

Statistical Methods

Differences between groups for BAL eosinophils, cytokine content in BALF and lung lymphocyte culture supernatant, and IgE were determined by analysis of variance. Statistical analyses for all tests were performed using a single value for each animal. All data are expressed as means ± standard error (SE). The number of animals per group was determined by power analysis using the following parameters: $\alpha = 0.05$, difference between groups = 50%, power = 0.8 (18).

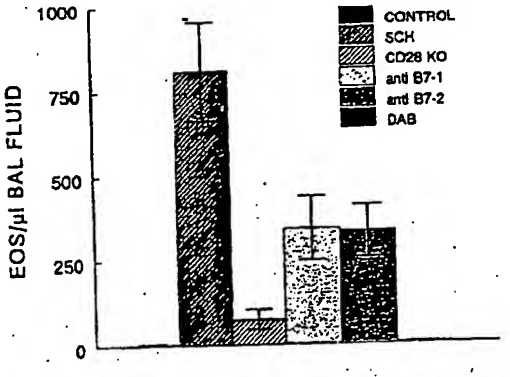


Figure 1. CD28/B7 interactions are required to generate eosinophil inflammation in *S. mansoni*–sensitized and airway-challenged mice. BAL was performed through a previously placed endotracheal tube. Four 0.8-ml aliquots of normal saline were infused, gently aspirated, and pooled. No eosinophils were recovered in BALF from the control group. Antigen challenge in *S. mansoni*–sensitized mice (SCH) resulted in dramatic BAL eosinophilia (807 ± 145 eos/μl BALF), which was profoundly inhibited in CD28KO mice (75 ± 29 eos/μl BALF, $P < 0.005$ versus SCH) or wild-type mice treated with anti-CD80 and anti-CD86 mAbs (DAB; 5 ± 2 eos/μl BALF, $P < 0.001$ versus SCH) beginning at antigen sensitization. Treatment with either anti-CD80 or anti-CD86 reduced BAL eosinophilia by 60% ($P < 0.05$ versus SCH for anti-CD80 or anti-B72). *Bars* represent the mean and SE of each group.

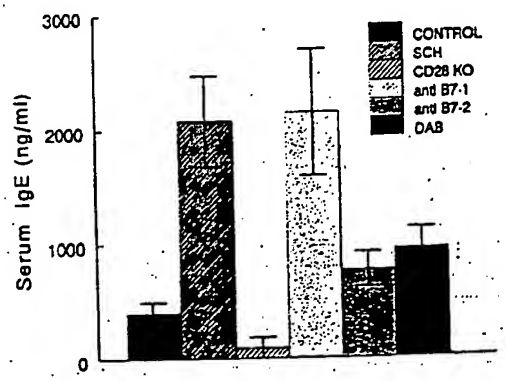


Figure 2. Anti-CD86 but not anti-CD80 treatment inhibits the systemic production of IgE. Serum was analyzed for IgE content by ELISA. IgE serum levels in SCH mice were increased almost 6-fold compared with CONTROL values (2,074 ± 394 ng/ml serum SCH versus 399 ± 94 ng/ml serum CONTROL). This was virtually abolished in CD28KO animals (92 ± 88 ng/ml serum, $P < 0.01$ versus SCH). Treatment with anti-CD86 significantly inhibited IgE production (768 ± 150 ng/ml serum, $P < 0.05$ versus SCH), and was equivalent to the effect produced by combined antibody therapy (double antibody [DAB], 950 ± 179 ng/ml serum, $P < 0.01$ versus SCH). In contrast, anti-CD80 treatment had no effect (2,148 ± 553 ng/ml serum).

Results

Effect of CD28 Gene Disruption on the Development of Atopic Airway Inflammation after *S. mansoni* Sensitization and Challenge

We have previously shown that mice sensitized and challenged with *S. mansoni* developed eosinophilic airway inflammation and airway hyperresponsiveness, and a Th2-like pattern of cytokine secretion; increased IL-5 and decreased IFN-γ in BALF, and increased IL-4 secreted from cultured lung lymphocytes *in vitro* (8). Administration of CTLA4Ig at a time of systemic antigen challenge prevented the development of the full allergic phenotype in this model, in part by causing immune deviation from a Th2-like cytokine response toward a Th1-like response. These earlier data, however, could not distinguish between the effects of CTLA4Ig in blocking CD28 versus CTLA-4 interactions. Further, we considered the possibility that CTLA4Ig might act directly on APCs through binding and crosslinking the B7 molecules (19). Previous studies from our group have shown that T cells isolated from CD28KO mice demonstrate similar abnormalities *in vitro* as do T cells stimulated in the presence of CTLA4Ig. T-cell proliferation, IL-2 production, and cell survival are diminished and, most significantly, Th2 responses are compromised (20). Therefore, CD28KO mice were examined in this *in vivo* model of atopic airway disease. As seen in Figure 1, a large number of eosinophils were found in BALF from SCH mice (807 ± 145 eos/μl BALF). In contrast, BAL eosinophilia in CD28KO mice (75 ± 29 eos/μl BALF, $P < 0.005$

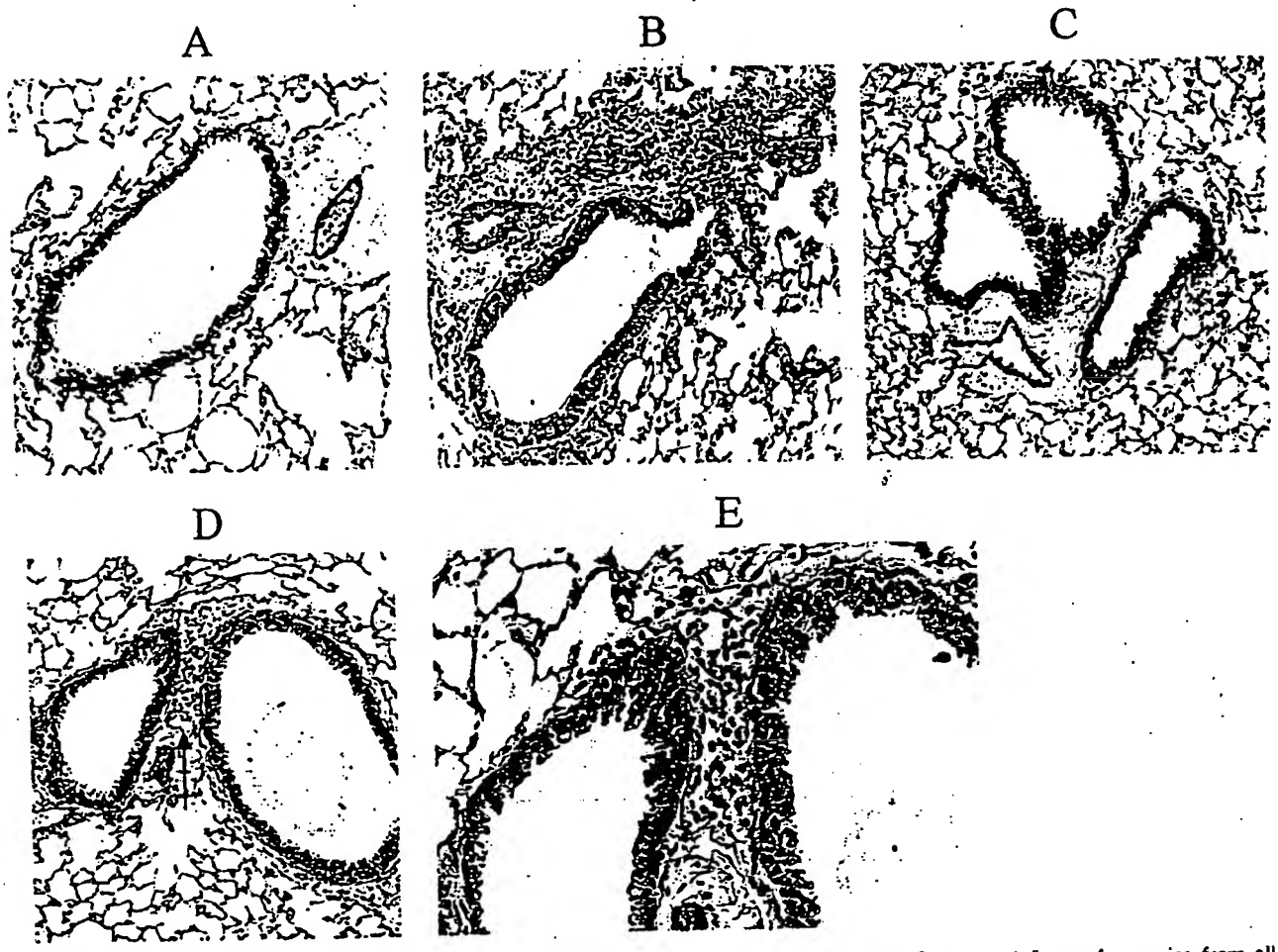


Figure 3. CD28KO mice developed mild airway lymphocytosis after antigen challenge with *S. mansoni*. Lungs from mice from all groups were fixed *in situ* with 10% formalin for 24 h and stained with H&E for routine analysis. (*A*) Lung from control mouse. There is no inflammatory infiltrate or edema around bronchioles. H&E stain, original magnification: ×400. (*B*) Lung from SCH mouse. There is a marked, predominantly eosinophilic infiltration and edema around bronchioles and vessels, with extension into the adjacent alveolar parenchyma. H&E stain, original magnification: ×400. (*C*) Lung from mouse treated with both anti-CD80 and anti-CD86 mAbs. There is minimal inflammation within and surrounding bronchioles and vessels. An occasional eosinophil is seen. H&E stain, original magnification: ×200. (*D*) Lung from CD28KO mouse. There is a mild peribronchiolar and perivascular lymphocytic infiltrate (*arrow*). Eosinophilic infiltration is not seen. H&E stain, original magnification: ×400. (*E*) High-power view of cellular infiltrate from *D*. H&E stain, original magnification: ×600.

TABLE 1

Effect of CTLA4Ig treatment on CD28KO mice sensitized and challenged with S. mansoni

	Wild-Type SCH ($n = 5$)	Wild-Type SCH + CTLA4Ig ($n = 5$)	CD28KO Control ($n = 3$)	CD28KO SCH ($n = 6$)	CD28KO SCH + CTLA4Ig ($n = 6$)
Eosinophils, % (BALF)	83 ± 1	49 ± 13	0	17 ± 4	15 ± 6
Total number of eosinophils (μl BALF)	331 ± 115	83 ± 38	0	9 ± 4	9 ± 4
Lymphocytes, % (BALF)	7 ± 2	12 ± 5	< 5	30 ± 6	17 ± 3
Total number of lymphocytes (μl BALF)	28 ± 6	20 ± 6	< 2	17 ± 7	10 ± 3
IL-4 (pg/ml BALF)	17 ± 1	13 ± 1	< 10	< 10	< 10
IL-5 (pg/ml BALF)	162 ± 24	59 ± 15	< 10	< 10	< 10
IFN-γ (ng/ml BALF)	1.15 ± 0.12	0.61 ± 0.09	0.64	0.91	0.83
Serum IgE (ng/ml)	3,447 ± 387	473 ± 158	ND	254 ± 21	257 ± 34
Proliferation (CPM; 2C11)	ND	ND	ND	14,588 ± 2,307	13,129 ± 2,130
Proliferation (CPM; SEA)	ND	ND	ND	8,495 ± 659	9,801 ± 880
(% cells) Thy 1.2+ CD25+	ND	ND	2.3 ± 0.2	5.5 ± 0.8	5.0 ± 0.2
(% cells) Thy 1.2+ CD69+	ND	ND	3.6 ± 0.7	6.2 ± 1.2	4.8 ± 0.4
(% cells) Thy 1.2+ CD44+	ND	ND	53.3 ± 1.9	54.0 ± 1.8	57.2 ± 2.9

ND: not determined.

versus wild type) was reduced by greater than 90%. Systemic IgE production was abolished in the CD28KO animals as well (2,074 ± 394 ng/ml IgE wild type versus 92 ± 88 ng/ml IgE for CD28KO, $P < 0.001$, Figure 2). However, the immune response was not totally ablated in these animals because a prominent population of lymphocytes was observed in the BALF. Although the total number of lymphocytes in BALF was not statistically significantly different, lymphocytes accounted for 30 ± 6% of all BAL cells in the CD28KO SCH group, compared with < 7% of BAL cells in isotype-treated CD28-KO or wild-type mice ($P < 0.05$; Table 1). These findings were confirmed histologically (Figures 3A–3D). Airways from SCH mice had consistent pathologic changes in airway epithelium and submucosa. Specifically, eosinophilic infiltration within epithelium and lamina propria and goblet-cell hyperplasia were never seen in control airways (Figure 3A) but were observed in many airways from all SCH mice (Figure 3B). Eosinophilic infiltration and mucus-cell hyperplasia were not seen in the CD28KO animals; however, there was a mild peribronchiolar lymphocytic infiltrate (Figures 3D and 3E). Thus, although SCH induced CD28-deficient mice to generate a lymphocytic infiltrate, there were no additional abnormal airway changes, including epithelial derangement or increases in goblet cells or submucosal glands.

The absence of eosinophils but presence of lymphocytes in the CD28KO mice suggested a significant alteration of Th1/Th2 balance in these sensitized and challenged mice. To access the cytokine milieu directly, BALF was extracted and evaluated for the presence of IL-5 and IFN-γ. The volume of recovered BALF was equivalent between groups and ranged from 2.5 to 2.8 ml. Unlike the BALF from control sensitized and challenged mice, the BALF from SCH CD28KO mice did not contain (Th2-derived) IL-5 in response to antigen challenge (159 ± 25 pg/ml IL-5 BALF wild type versus < 10 pg/ml IL-5 BALF for CD28KO; Figure 4A). The absence of IL-5 might explain the reduction of eosinophilia in the CD28KO mice, although additional factors—including a reduction in IL-4 and the possible inhibition of chemokine-directed eosinophilotaxis—may play significant roles as well. Interestingly, secretion of the prototype Th1 cytokine (IFN-γ) in BALF from CD28KO animals was equal to wild-type mice (0.96 ± 0.2 ng/ml IFN-γ BALF CD28KO versus 0.91 ± 0.11 ng/ml IFN-γ BALF wild type, Figure 4B). Thus, our data support an essential role for CD28 signaling in the development of eosinophilia and production of Th2-type cytokines by the infiltrating BAL T cells.

Effects of Anti-CD80 and Anti-CD86 Treatment in the Development of Allergic Airway Inflammation

Mice were treated from the outset with a combination of both anti-CD80 and anti-CD86 mAbs. As seen in Figure 1, there were virtually no eosinophils (5 eos/μl) in BALF in mice treated with both anti-B7 mAbs. Cytokine analysis of the BALF confirmed the suppression of the Th2 responses in mAb-treated mice. Little IL-5 in BALF (25 ± 10 pg/ml versus 159 ± 25 pg/ml for SCH mice) was observed in treated animals (Figure 4A). IFN-γ production was also greatly reduced (0.211 ± 0.04 ng/ml versus 0.91 ± 0.11 ng/ml SCH, $P < 0.005$) in BALF from mice treated with both mAbs (Figure 4B). Finally, combined antibody treatment greatly decreased serum IgE responses (950 ± 179 ng/ml versus 2,074 ± 394 ng/ml for SCH, $P < 0.01$, Figure 2) and blocked all histologic evidence of the disease (Figure 3C).

Most *in vivo* studies have suggested that CD86 is critical for initiating immune responses and is essential for Th2 development (5). However, CD80 has been found to play an important role in the late stages of some Th2-mediated disorders, including autoimmune disease (21). The role of CD80 and CD86 in asthma is also controversial. For example, Harris and colleagues determined that CD80 ligation was critical for development of allergic airway inflammation (22). In contrast, other studies have suggested that CD86 plays a dominant role in the development of murine asthma (23, 24). Therefore, we examined the effects of anti-B7 mAbs individually beginning at the time of antigen sensitization. Treatment with either anti-CD80 or anti-

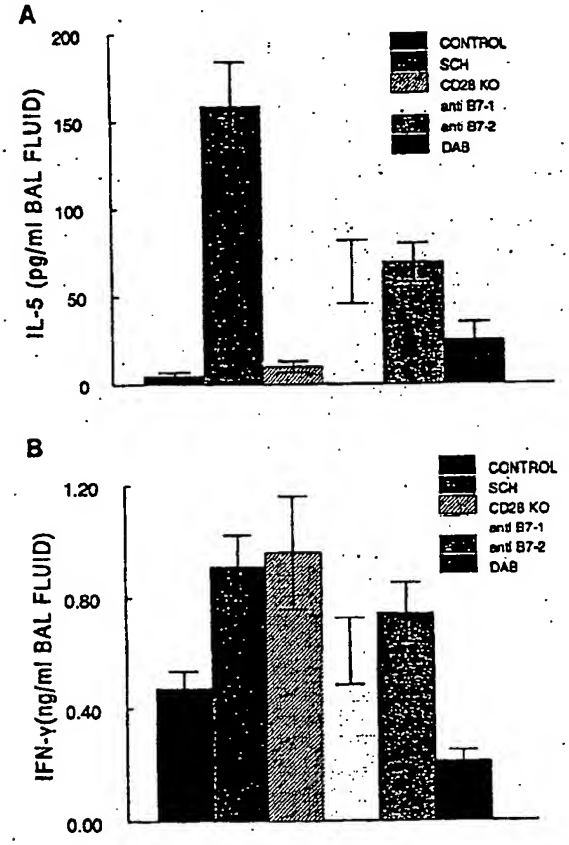


Figure 4. (*A*) IL-5 production is dramatically reduced in CD28-KO mice and wild-type mice treated with anti-B7 antibodies. Cell-free BALF was evaluated for IL-5 and IFN-γ content, respectively, by ELISA. CONTROL mice produced minimal IL-5 (5 ± 1 pg/ml BALF). In contrast, SCH mice produced large amounts of this cytokine (159 ± 25 pg IL-5/ml BALF). Secretion of IL-5 was abolished in CD28KO mice (10 ± 3 pg IL-5/ml BALF, $P < 0.001$ versus SCH) and was dramatically inhibited in animals treated with both anti-B7 antibodies (25 ± 10 pg IL-5/ml BALF, $P < 0.005$ versus SCH). Either anti-CD80 or anti-CD86 antibody treatment inhibited secretion of this "Th-2–like" cytokine by > 60% (64 ± 18 pg/ml BALF and 69 ± 11 pg/ml BALF, respectively, $P < 0.05$ versus SCH). (*B*) IFN-γ production was not affected by genetic deletion of CD28. CD28KO and wild-type mice secreted equivalent amounts of IFN-γ into BALF (0.96 ± 0.2 ng/ml BALF, CD28KO; 0.91 ± 0.11 ng/ml BALF, SCH). Treatment with individual anti-B7 antibodies had no significant effect on BALF IFN-γ content (0.61 ± 0.24 ng/ml BALF, anti-CD80; 0.74 ± 0.15 ng/ml BALF, anti-CD86). However, treatment with both antibodies (DAB) reduced BALF IFN-γ content by 75% (0.211 ± 0.04 ng/ml BALF, $P < 0.005$ versus SCH).

CD86 mAb from Day 0 resulted in significantly reduced airway eosinophilia (Figure 1) (341 ± 94 eos/μl BALF anti-CD80–treated mice, $P < 0.01$ versus SCH; 332 ± 76 eos/μl BALF anti-CD86–treated mice, $P < 0.01$ versus SCH). These findings were confirmed by histologic examination revealing that treatment with either anti-B7 mAb reduced but did not abolish airway tissue eosinophilia. Inhibition of either CD80 or CD86 ligation also reduced, but did not completely inhibit, secretion of IL-5 (64 ± 18 pg/ml BALF and 69 ± 11 pg/ml BALF, respectively, $P < 0.05$ versus SCH; Figure 4A). Interestingly, anti-CD86 but not anti-CD80 antibody treatment attenuated production of IgE in serum (768 ± 150 ng/ml BALF and 2,148 ± 553 ng/ml BALF, respectively, versus 2,074 ± 394 ng/ml BALF for SCH group, $P < 0.05$ versus anti-CD86–treated group, Figure 2). Moreover, the level of IgE suppression was equivalent to that observed in the group treated with the combination of anti-CD80 and anti-CD86 mAbs. Finally, IFN-γ secretion into BALF was not significantly affected by treatment with antibody against either CD80 or CD86 (Figure 4B). Together, these results suggest that the ligation of CD28 with either B7 molecule is sufficient to induce a partial local Th2-type cytokine response. However, CD86 ligation, not CD80, is essential for the induction of a systemic IgE response.

Effects of CTLA4Ig Treatment on *S. mansoni*–Sensitized and Challenged CD28KO Mice

In contrast to animals treated with both anti-B7 antibodies, the CD28KO mice generated significant IFN-γ in BALF, and developed mild lymphocytic inflammation of airways. Therefore, to test the possibility that an alternative T-cell surface molecule may interact with B7 to costimulate this lymphocytic response, we treated an additional six CD-28KO mice with CTLA4Ig, beginning at sensitization. This soluble chimeric fusion protein binds CD80 and CD86 with a 20-fold greater avidity compared with CD28 (25). Five wild-type animals were sensitized and challenged to serve as controls, and an additional five wild-type animals were treated with CTLA4Ig to confirm the efficacy of this drug to block CD28/B7 interactions. As expected, wild-type animals generated an allergic phenotype that was greatly inhibited by pretreatment with CTLA4Ig. However, in the CD-28KO mice CTLA4Ig treatment had no significant effect on airway lymphocytosis (30 ± 6% lymphs/BALF, isotype-treated versus 17 ± 3 lymphs/BALF, CTLA4Ig-treated, P = ns). Additionally, spleen cells from SCH CD28KO mice treated with CTLA4Ig or isotype control proliferated in an equivalent manner in response to 2C11 and SEA, and had similar surface expression of CD25, CD44, and CD69 (Table 1). Thus, engagement of B7 with an alternative molecule(s) including CTLA-4 could not explain the different results obtained from CD28KO and anti-CD80/anti-CD86–treated wild-type animals.

Effects of Anti-CD80 and Anti-CD86 Treatment during the Challenge Phase of Allergic Airway Inflammation

We next evaluated the effect(s) of treatment with anti-B7 antibodies when administered after antigen sensitization and during antigen challenge inasmuch as this setting might more closely approximate the clinical situation in which already-sensitized patients become symptomatic upon exposure to the sensitizing antigen. Mice were given *S. mansoni* as described and treated with either anti-CD80 or anti-CD86 or both mAbs beginning at the time of antigen challenge (daily from Days 7 to 10). Treatment with either mAb alone had no significant effect on any of the disease parameters. However, the combination of both mAbs during the challenge period resulted in a 75% reduction in airway eosinophilia and IL-4 and IL-5 production in BALF. Interest-

TABLE 2

Effect of anti-B7 mAb treatment given during antigen challenge (Days 7 to 10) in S. mansoni*–sensitized mice*

	% EOS BALF	TOTAL EOS (μl) BALF	IL-4 (pg/ml) BALF	IL-5 (pg/ml) BALF	IFN-γ (ng/ml) BALF	IgE (ng/ml) Serum
Control (n = 8)	< 1	< 1	< 3	< 5		565 ± 241
SCH (n = 15)	70 ± 3	250 ± 33	54 ± 8	186 ± 31	1.15 ± 0.08	1,586 ± 162
Anti–B7-1 Days 7 to 10 (n = 10)	69 ± 5	214 ± 45	58 ± 9	140 ± 25	1.11 ± 0.09	1,519 ± 433
Anti–B7-2 Days 7 to 10 (n = 10)	57 ± 9	135 ± 53	36 ± 6	97 ± 18	0.90 ± 0.07	1,230 ± 254
DAB Days 7 to 10 (n = 10)	42 ± 9	67 ± 28*	18 ± 6†	49 ± 13‡	0.93 ± 0.09	1,487 ± 253

EOS: eosinophils.
* $P < 0.001$ versus SCH.
† $P = 0.03$ versus SCH.
‡ $P = 0.02$ versus SCH.

ingly, production of IFN-γ in the BALF and serum IgE levels were equivalent to those in SCH mice (Table 2).

Expression of CD80 and CD86 in SCH Mouse Lung

CD86 is generally assumed to play a dominant role early in the course of T-cell activation. However, the relative effectiveness of anti-CD80 mAb in the local immune responses in the lung, especially late in the disease progression, suggested that this costimulatory ligand might be selectively upregulated in lung tissue. Thus, we examined the expression of CD80 and CD86 mRNA in lung tissue from SCH mice, beginning at a time point from sensitization through 72 h after antigen challenge. We found that both CD80 and CD86 mRNA (not shown) were detected in the lungs of unmanipulated mice as well as in SCH mice examined after challenge with SEA. Next, surface expression of both B7 molecules on collagenase-digested lung cells was assessed by FACS analyses at equivalent time points after antigen challenge in SCH mice (Figure 5). CD80 surface expression was constitutive (median fluorescence intensity [MFI] 6 ± 1), was upregulated within 24 h (MFI = 19 ± 1), and continued to be significantly expressed at 72 h after antigen challenge (MFI = 15 ± 1). Surface expression of CD86 was also constitutive (MFI = 11 ± 2) and elevated within 24 h (MFI = 21 ± 2), although expression returned to baseline levels by 72 h after antigen challenge (MFI = 12 ± 1).

Of particular interest was the finding that eosinophils expressed primarily CD80 (26% of all cells) versus CD86 (10% of all cells; Figure 6) at 72 h. In contrast, alveolar macrophages (data not shown) and lymphocytes expressed primarily CD86 (28% of all cells), with lesser expression of CD80 (7% of all cells) at this time point. We are aware of one previous study that addressed B7 expression on eosinophils. In this study, the authors demonstrated upregulation of both CD80 and CD86 expression on eosinophils from peritoneal exudate of IL-5 transgenic mice after stimulation with granulocyte macrophage colony-stimulating factor (26). Thus our data again emphasize the potential differences in B7 expression on different cells in different immune environments. Importantly, these data also raise the possibility that eosinophils may provide additional costimulatory signals in a rapidly developing allergic environment, thus potentially making the allergic lung a somewhat unique organ in terms of expression of B7 costimulatory molecules.

Discussion

The results of the current study illustrate the central role of CD28 in the induction of allergic airway responses in *S. mansoni*–sensitized and airway-challenged mice. These animals develop an atopic respiratory phenotype including airway eosinophilia and goblet-cell hyperplasia, secrete IL-4 and IL-5 locally within the respiratory tract, and produce significant amounts of systemic IgE. Either CD28-deficient mice or combined treatment with anti-CD80 and anti-CD86 mAbs completely suppressed the Th2-driven allergic airway inflammatory response, whereas treatment with either anti-CD80 or anti-CD86 mAbs had an equivalent effect to partially suppress allergic inflammation. In addition, simultaneous treatment of sensitized mice at the time of antigen challenge with both anti-CD80 and anti-CD86 mAbs significantly inhibited the development of airway eosinophilia, histologic evidence of mucus cell hyperplasia/hypertrophy, and the secretion of IL-4 and IL-5 in BALF. We have previously reported that treatment with CTLA4Ig after antigen sensitization blocked the development of airway eosinophilia, airway hyperresponsiveness, and production of Th2-like cytokines and systemic IgE in *S. mansoni*–sensitized and challenged mice (8). Thus, our present findings suggest that blocking CD28/B7 interactions either before or during antigenic challenge inhibits the development of Th2-like lymphocytes. Because asthma is not reliably predicted in asymptomatic people, the treatment of asthmatic patients is initiated after primary immunization with antigen. Thus, our current data imply that inhibiting the T-lymphocyte costimulation pathway in sensitized individuals may be a potentially worthwhile therapeutic strategy to treat people with atopic respiratory disorders. However, our findings also suggest that strategies that are designed to inhibit CD28/B7 interactions should be based on the particular individual kinetics of expression of CD80 and CD86 in human lung tissue.

Although our results suggest that CD28 ligation is necessary for production of Th2 cells, sensitized CD28KO mice produced significant IFN-γ and developed a mild lymphocytosis in response to antigen challenge. This was in contrast to wild-type mice treated with mAbs to both B7 molecules, in which IFN-γ production and lymphocyte infiltrates were both inhibited. These data are similar to those reported by Brown and associates (27) in which T cells from CD28KO mice on either a C57BL/6 or BALB/c

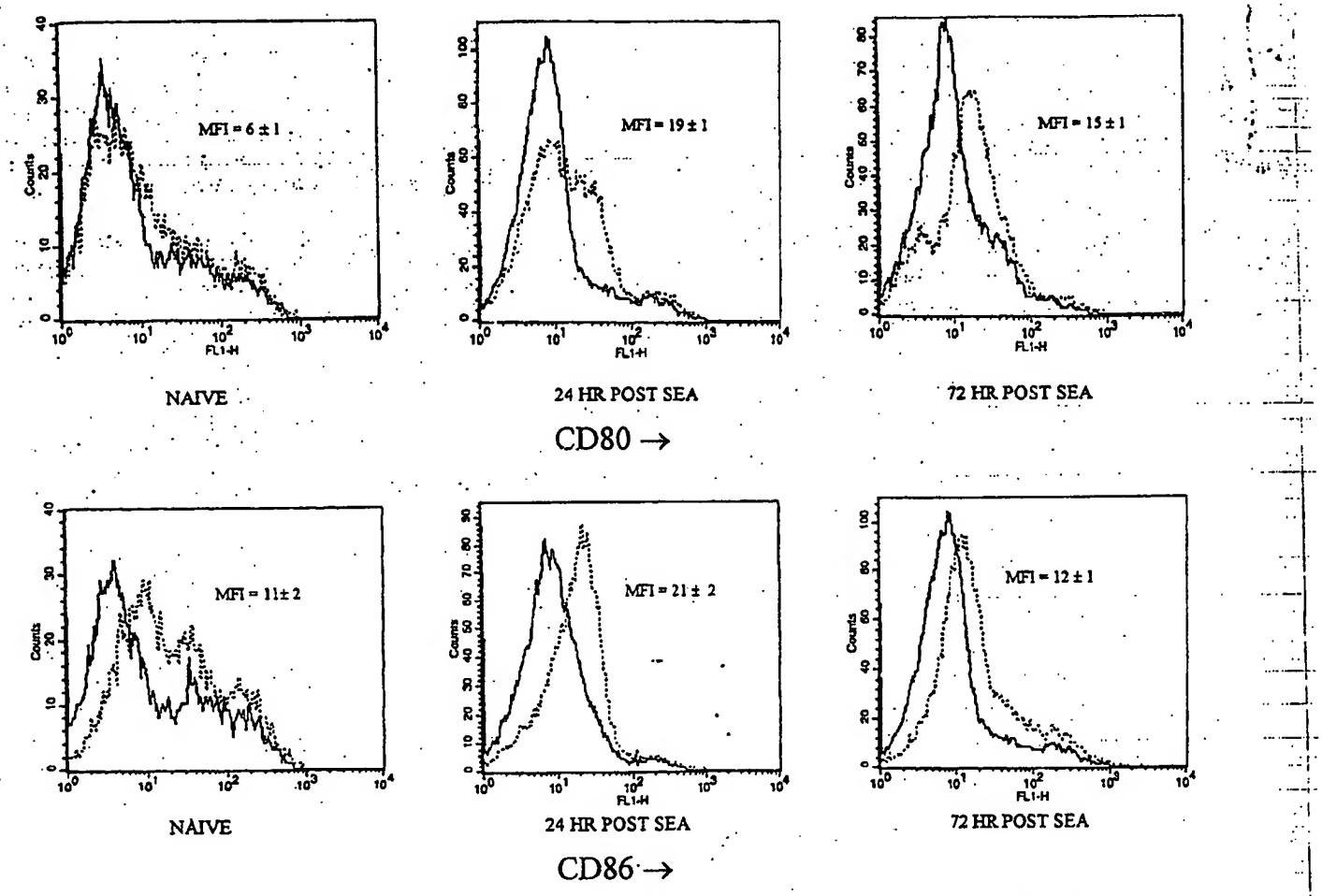


Figure 5. Representative FACS analysis of B7 expression on whole lung cells from antigen-sensitized and challenged mice. Histograms derived from a scatter plot using gated, live cells obtained by collagenase digestion of whole lung from *S. mansoni*–sensitized mice before (naive) and 24 and 72 h after antigen challenge. Histograms represent unstained cells (*solid lines*) or cells stained with anti–CD80 fluorescein isothiocyanate (FITC) (*dashed lines*, CD80) or anti–CD86 FITC (*dashed lines*, CD86) at the time points indicated. Lung cells from CD80KO and CD86KO mice were used as negative controls (not shown). Data are representative of four to seven experiments for each time point.

background produced IFN-γ in response to infection with *Leishmania major* when compared with wild-type controls. These data also support findings from our lab and others (10) demonstrating that CD28KO mice can mount an efficient, although delayed, Th1 rejection response after organ transplantation.

The explanation for these differing results obtained in the CD28KO mouse and in wild-type mice treated with anti-CD80 and anti-CD86 mAbs is not clear. We considered the possibility that there may be an additional B7-dependent costimulatory ligand(s) on T cells responsible for induction of partial T-cell activation. However, we observed that blockade of B7 ligation with CTLA4Ig did not affect the generation of IFN-γ or the development of a mild lymphocytic infiltrate in these sensitized and challenged CD28KO mice. Thus, it is more likely that the data reflect intrinsic differences between antibody treatment of the CD28/B7 pathways and gene disruption of CD28. In this regard, heart allograft rejection that is fully blocked by CTLA4Ig is not prevented in CD28KO mice even after CTLA4Ig therapy. In fact, blocking CTLA-4/B7 interactions accelerates Th1-mediated allograft rejection in CD28-deficient mice (10). Moreover, we have demonstrated that T cells from CD28KO mice expand significantly before activation-induced cell death (unlike cells cultured with CTLA4Ig [28]). We speculate that the absence of CD28 expression may result in a subtle alteration in T-cell development (29, 30) or changes in mature T-cell signal transduction due to the absence of cell surface membrane–expressed CD28 that can interact with intracellular signaling molecules such as ITK (31) or PI3 kinase (32).

Our data also raise several questions with regard to the individual role of the B7 molecules and the importance of the temporal expression of these costimulatory ligands in

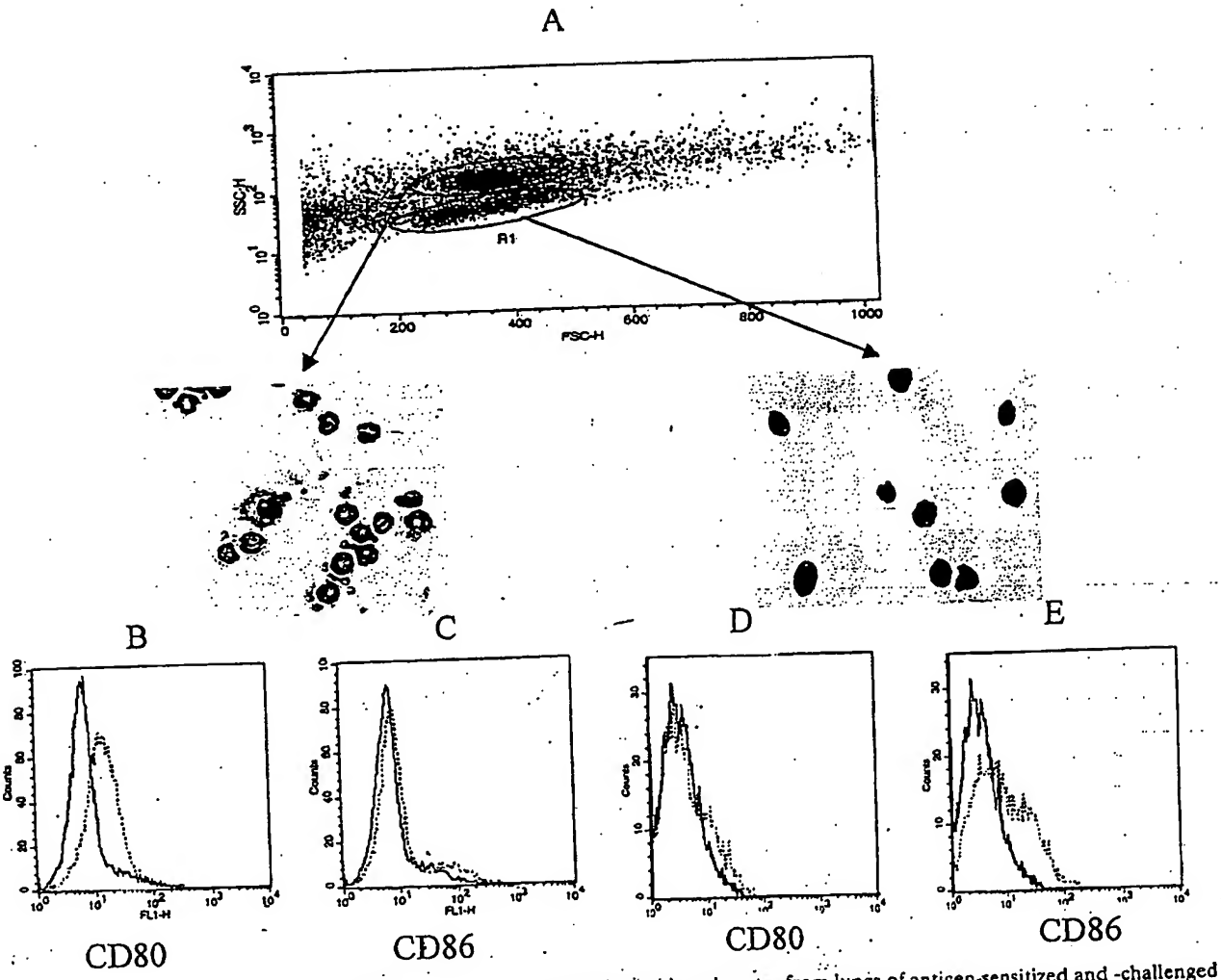


Figure 6. Flow cytometric detection of B7 expression on eosinophils and lymphocytes from lungs of antigen-sensitized and -challenged mice. Scatter plot (*A*) of cells obtained by collagenase digestion of whole lung from *S. mansoni*–sensitized mice 72 h after injection of antigen into trachea. The gated regions were sorted and contained 95% eosinophils (R2, < 1% eosinophils at baseline) or lymphocytes (R1), respectively, at 72 h. Eosinophils stained positively with FITC-conjugated anti-CD80 (*B, dashed lines*, 26% of all cells) and FITC-conjugated anti-CD86 (*C, dashed lines*, 10% of all cells) compared with FITC-conjugated control mAb (*B* and *C, solid lines*). For comparison, B7 staining on lymphocytes was somewhat different, with 7% of all cells staining positively for CD80 (*D*) versus 28% positive for CD86 (*E*). Data are representative of four to seven experiments.

promoting T-cell effector functions *in vivo*. Specifically, in the current study, antibody treatment directed against either CD80 or CD86 alone had a partial and similar inhibitory effect on the development of allergic inflammation. Although several studies have reported that CTLA4Ig can block the development of allergic airways in small-animal models, the individual roles of CD80 and CD86 have remained controversial. For example, Harris and coworkers have described the requirement for CD80 but not CD86 in the induction of murine allergic airway inflammation (22). In apparent contradiction, two additional studies reported that anti-CD86 treatment of ovalbumin (OVA)-sensitized mice immediately before antigen challenge resulted in greatly diminished airway eosinophilia and airway hyperresponsiveness (23, 24). However, in both studies eosinophilic recruitment into airways was also significantly reduced in mice treated with only the anti-CD80 antibody.

Perhaps not surprisingly, CD80 and CD86 play significantly different roles in determining immune responses *in vivo* in other, nonasthma models as well (33). For example, we and others have shown that selective inhibition of B7 ligands can alter the disease state in an animal model of diabetes. Non-obese diabetic mice treated with anti-CD86 antibodies had a decreased incidence of diabetes, whereas treatment with anti-CD80 resulted in the opposite effect (34). In contrast, CD80 appeared to be the dominant costimulatory ligand in regulating experimental autoimmune encephalitis (EAE) relapses (35). We believe that these different results may be explained, in part, by the kinetics of expression of CD80 and CD86 on individual cells in individual

organs during antigen sensitization. In this context, murine (C57BL/6) lung dendritic cells constitutively express both B7 ligands. Masten and associates have recently shown in this system that CD80 is the primary ligand needed to stimulate dendritic cell–initiated allogeneic T-cell proliferation (36). However, if dendritic cells were the essential APCs in allergic mouse lung, we would anticipate that anti-CD80 mAb treatment would have had a greater effect to inhibit airway eosinophilia and IL-4 and IL-5 secretion compared with anti-CD86 mAb treatment. Yet in our system the two individual mAb treatments had equivalent local effects *in vivo*. This suggests that multiple cell types may participate to present B7 molecules in the development of murine allergic airways. Additionally, in the current study we demonstrate by reverse transcriptase/PCR and FACS analysis that both CD80 and CD86 were expressed on lung cells from naive animals and were upregulated within 24 h of antigen challenge. We also found that both B7 molecules were expressed on eosinophils from the allergic lung and that there was greater expression of CD80 than CD86 on these inflammatory cells.

Thus, B7 expression on lung cells in this animal model of respiratory atopy does not conform to the paradigm of minimal constitutive expression and antigen-induced "early" CD86, "late" CD80 expression originally described for human B cells (37). This further suggests that expression of B7 molecules on potential APCs varies depending not only on the time they are examined and on the organ from which the cells are harvested, but also on the local environment in which the animals are kept. Specifically, eosinophils are typically not present in nonallergic tissues, therefore asthma and atopic diseases may represent a somewhat unique setting for eosinophils to function as presenting cells for costimulatory molecules. We speculate that this population of cells may act as APCs in this system to support and augment the rapidly increasing requirements for T-cell costimulation during the initial phases of an allergic response in airways. It is also likely that resident (noneosinophil) cells within the pulmonary system of "naive" animals encounter nominal antigen even though the animals have not been manipulated experimentally.

Our results are most consistent with the model that either of the B7 molecules can act as a costimulatory ligand to initiate immune reactions, but that the temporal kinetics and level of expression of either CD80 or CD86 determines the ability of either of these molecules to influence T-cell differentiation and effector functions (38). For example, Schweitzer and colleagues reported that the costimulatory signals provided by CD80 and CD86 were basically equivalent because both could elicit IL-4 as well as IFN-γ secretion by anti-CD3–stimulated CD4+ T cells, especially under suboptimal conditions (39). Similarly, *Heligmosomoides polygyrus*–infected mice seem relatively resistant to the effect of treatment with antibodies to either B7 molecule. However, treatment with antibodies to both B7 ligands could block the immune and inflammatory reactions in these animals (40). Previously, we have shown that anti-CD86 treatment exacerbates the developing stages of EAE whereas anti-CD80 treatment exacerbates already-established EAE. The different effects of anti-B7 treatment in this model are due to increased early expression of CD86 within the central nervous system followed by dominant CD80 expression later as the disease progresses (21). Thus, our results suggest that the "dominance" of either B7 molecule in "Th1" or "Th2" immune responses can be explained by a more rapid upregulation after antigen presentation (39). Our findings may also offer a temporal and functional explanation for previous studies in which CD80 or CD86 seem to play different, sometimes mutually exclusive roles in Th1/Th2 production and disease progression (11, 15, 21–24, 33, 35, 38–40).

Although treatment with anti-CD80 or anti-CD86 resulted in partial reduction in the development of allergic airways, only anti-CD86 treatment significantly depressed systemic production of IgE. These data are consistent with a previous study by Harris and associates in which CD80 blockade prevented the development of allergic airways but did not inhibit systemic production of IgE in mice (22). Treatment with anti-CD86 mAbs has previously been shown to result in deficiencies in germinal center formation and isotype switching (41). CD86- but not CD80-dependent immunoglobulin production has also been demonstrated in SV129 OVA-challenged mice (24) and the lupus-prone NZW mouse (42). Thus, while local (pulmonary) lymphocyte responses to antigen may be driven equally by CD28/CD80 or CD28/CD86 ligation, CD86 seemingly plays the predominant role in the systemic B-cell response in the mouse. This implies that constitutive expression of both CD80 and CD86 in lung may be different than in peripheral lymph node germinal centers and Peyers patches, regions that play a significant role in systemic IgE responses and where CD86 may be more dominantly expressed. We speculate that the presence of CD80-expressing eosinophils in the allergic lung but not in nonpulmonary tissues might account for these differences.

These potential regional differences in B7 expression have significant implications for developing clinical treatment strategies. Specifically, therapy for asthma that targets CD28/B7 interactions may need to consider the potential for differential expression of B7 molecules in different organs and immune environments in the same individual. This important point has recently been elegantly demonstrated in a murine model of relapsing encephalomyelitis (38). Finally, CD28/B7 inhibition after sensitization and during antigen challenge inhibited most of the allergic lung response, suggesting that targeted manipulation of the second signal involved in T-cell activation may lead to effective treatments for people with allergic respiratory disorders, including atopic asthma.

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